

**Remarks/Arguments** follow the amendment sections of this paper.

**AMENDMENTS TO THE SPECIFICATION:**

**Replace the paragraph spanning page 13, line 21 to page 14, line 6 with the following paragraph:**

The present invention is ~~now~~ described and illustrated by the following experiments in specific embodiments illustrated by drawings in which:-

- Exp. Fig. 1 shows gp130-dependent phosphorylation of SHP-2 in ES cells;
- Exp. Fig. 2 shows effect of mutating tyrosine 118 on gp130-dependent self-renewal and growth of ES cells;
- Exp. Fig. 3 shows gp130-dependent phosphorylation of ERK1 and ERK2 in ES cells;
- Exp. Fig. 4 shows effect of the MEK inhibitor, PD098059, on ES cell self-renewal and ERK activation;
- Exp. Fig. 5 shows effect of PD098059 on ES cell pluripotency;
- Exp. Fig. 6 shows decay of activated STAT3 following stimulation of gp130 and the chimeric GRgp130 receptors;
- Exp. Fig. 7 shows gp130-dependent induction of SOCS-3 gene expression in ES cells; and
- Exp. Fig. 8 shows the effect of an MEK inhibitor on stem cell differentiation.

**Replace the paragraph spanning page 14, lines 1 to 17 with the following paragraph:**

Exp.1 demonstrated ~~In more detail, Fig.1 shows~~ gp130-dependent phosphorylation of SHP-2 in ES cells. ES cells expressing either the GRgp(278) or GRgp(Y118F) chimeric receptors were induced with IL-6 (100 ng/ml plus sIL-6R) or G-CSF (30 ng/ml) for 15 minutes. SHP-2 protein was immunoprecipitated from lysates of unstimulated or stimulated cells, fractionated on a SDS-polyacrylamide gel and transferred to a

nitrocellulose membrane. The filter was probed with anti-phosphotyrosine antibody (upper panel), stripped and reprobed with anti-SHP-2 antibody (lower panel). The position of tyrosine phosphorylated forms of SHP-2 and two additional proteins was determined are indicated by arrows.

**Replace the paragraph spanning page 14, line 19 to page 15, line 2 with the following paragraph:**

Exp. 2 demonstrated Fig.2 shows the effect of mutating tyrosine 118 on gp130-dependent self-renewal and growth of ES cells including stem cells:

———(A) Stem cell renewal was mediated by GRgp(278) and GRgp(Y118F) chimeric receptors in response to G-CSF. Self-renewal, as measured by  $\beta$ -galactosidase expression from the Oct-4 locus was assayed after 6 days in culture with G-CSF (300 fg-30 ng/ml). Data for two independent clones are represented as means  $\pm$  s.e.m. for duplicate determinations of triplicate samples normalized relative to the response with IL-6(100 ng/ml plus sIL-6R).

———(B) Photomicrographs were taken of X-gal stained, representative colonies formed by GRgp(278) and GRgp(Y118F) transfectants after 6 days culture with 300 fg, 30 pg and 30 ng/ml of G-CSF.

———(C) Photomicrographs were also taken of representative colonies formed by GRgp(278) and GRgp(Y118F) transfectants after 6 days culture with no cytokine, IL-6 (100 ng/ml + sIL-6R), G-CSF(30 ng/ml) or , IL-6 (100 ng/ml + sIL-6R) and G-CSF(30 ng/ml).

**Replace the paragraph spanning page 15, lines 4 to 12 with the following paragraph:**

Exp. 3 demonstrated Fig.3 shows gp130-dependent phosphorylation of ERK1 and ERK2 in ES cells. ES cells expressing either the GRgp(278) or GRgp(Y118F) chimeric receptors were either untreated or stimulated with IL-6(100 ng/ml plus sIL-6R) or G-CSF (30 ng/ml) for 10 or 20 minutes. Cell lysates were separated on a 10% SDS-acrylamide gel,

electroblotted onto a nitrocellulose membrane and probed sequentially with antibodies specific for the active phosphorylated form of ERK and STAT3. Reprobing the stripped filter with an antibody that binds to both phosphorylated and dephosphorylated ERKs verified that equivalent amounts of protein were loaded in the samples.

**Replace the paragraph spanning page 15, line 14 to page 16, line 2 with the following paragraph:**

Exp. 4 demonstrated ~~Fig. 4 shows~~ the effect of the MEK inhibitor, PD098059, on ES cell self-renewal and ERK activation. ~~To demonstrate self activation:~~

~~——(A) Self-renewal of ES cells treated with PD098059.~~ PD098059. D027 ES cells grown at a subsaturating level of LIF (5 U/ml) were treated with PD098059 for 5 days and assayed for  $\beta$ -galactosidase expression from the *Oct-4* locus. Data were collected as ~~are~~ means  $\pm$  s.e.m for duplicate determinations of triplicate samples normalized relative to the response to LIF. To demonstrate

~~——(B) PD098059 dependent inhibition of ERK activation.~~ ~~activation.~~ GRgp(278) transfected D027 cells were cultured with a subsaturating level of LIF (5 U/ml) and PD098059 for 48 hours. Cells were then stimulated with G-CSF (30 ng/ml) for 10 minutes, lysed in sample buffer and analyzed for ERK activation by immunoblotting with phosphospecific anti-ERK antibodies. Subsequent probing of the filter with an antibody that binds to both phosphorylated and dephosphorylated ERKs confirmed that equivalent amounts of protein were loaded in all samples. To demonstrate the effect

~~——(C) Effect of PD098059 on the dose response of ES cells to LIF, the LIF.~~ The dose response of D027 ES cells to LIF in 25  $\mu$ M PD098059 or vehicle (0.05% DMSO) was measured by  $\beta$ -galactosidase expression from the *Oct-4* locus. Data was collected as ~~are~~ means  $\pm$  s.e.m for duplicate determinations of triplicate samples normalized relative to the maximum response of cells to treatment with LIF(111 U/ml) plus vehicle.

**Replace the paragraph spanning page 16, lines 4 to 9 with the following paragraph:**

Exp. 5 demonstrated ~~Fig. 5 shows~~ the effect of PD098059 on ES cell pluripotency. ZIN40 ES cells were treated with 25  $\mu$ M PD098059 plus 5 U/ml LIF for 48 hours, re-fed with medium containing LIF for a further 24 hours and then microinjected into C57BL/6 blastocysts. Embryos were collected at day 9.5 of pregnancy and stained for  $\beta$ -galactosidase activity. ~~Representative embryos are shown in the panel.~~

**Replace the paragraph spanning page 16, lines 11 to 20 with the following paragraph:**

Exp. 6 demonstrated ~~Fig. 6 shows~~ decay of activated STAT3 following stimulation of gp130 and the chimeric GRgp130 receptors. GRgp(278) and GRgp(Y118F) ES cell transfectants were stimulated with IL-6 (100 ng/ml plus sIL-6R) or G-CSF (30 ng/ml) for 25 minutes (0\*), re-fed with cytokine free medium and samples were collected at 40 minute intervals. Immunoblots of cell lysates were probed sequentially with an antibody specific for the active phosphorylated form of STAT3 and then with an antibody that recognizes both phosphorylated and unphosphorylated STAT3. Activation of STAT3 was ~~Note that activation of STAT3 is~~ associated with the appearance of a slower migrating STAT3 species, presumed to be the serine phosphorylated form of STAT3.

**Replace the paragraph spanning page 16, line 21 to page 17, line 2 with the following paragraph:**

Exp. 7 demonstrated ~~Fig. 7 shows~~ gp130-dependent induction of SOCS-3 gene expression in ES cells.

~~——(A) Northern analysis was performed on total RNA (10 g) prepared from ES cells expressing the GRgp(278), GRgp(Y126-275F) and GRgp(Y118F) receptors, unstimulated (-) or stimulated either with LIF (L, 100 units/ml) or G-CSF (G, 30 ng/ml) for the indicated times (minutes). Hybridisation of the  $\approx$  3 kb SOCS-3 mRNA and ethidium bromide staining of the 18S rRNA was observed is shown in the upper and lower three panels, respectively.~~

~~——(B) Graphic representation of SOCS-3 mRNA expression shown in panel A. SOCS-3 mRNA was expressed and~~ hybridization was quantitated by phosphorimage analysis

and signals were normalized relative to those obtained in each cell line at 90 minutes after stimulation with LIF.

**Replace the paragraph spanning page 17, lines 4 to 5 with the following paragraph:**

Exp. 8 demonstrated ~~Fig. 8 shows~~ that an MEK inhibitor of the invention sustains undifferentiated ES cells in an aggregate culture.

**Replace the paragraph spanning page 21, lines 14 to 25 with the following paragraph:**

The data collected from Exp. 2 demonstrated ~~presented in Figure 2A shows~~ that self-renewal of GRgp(278) transfectants increased in a dose dependent manner, reaching a plateau at 3-30 ng/ml G-CSF. In contrast, the maximal self-renewal response of GRgp(Y118F) ES cells was achieved at just 30 pg/ml G-CSF. The morphology of the GRgp(Y118F) colonies maintained in 30 pg/ml G-CSF was typical of undifferentiated ES cells ~~(Figure 2B)~~. This result establishes that activation of SHP-2 through tyrosine 118 is not required to direct ES cell self-renewal. Equivalent levels of both receptor chimaeras were expressed at the cell surface of ES cell transfectants, as judged by binding studies with <sup>125</sup>I-labelled G-CSF ~~(data not shown)~~. Therefore the shift in dose response suggests that the mutant receptor may have enhanced signaling activity.

**Replace the paragraph spanning page 21, line 27 to page 22, line 4 with the following paragraph:**

Interestingly, at higher concentrations of G-CSF, GRgp(Y118F) transfectants formed small aggregates of cells rather than the more flattened colony morphology normally associated with undifferentiated ES cells ~~(Figure 2B)~~. These colonies expressed  $\beta$ -galactosidase and stained positive for the stem cell marker alkaline phosphatase ~~(Figure 2B and data not shown)~~, indicating that the ES cells remained undifferentiated. This was confirmed by the resumption of typical ES cell growth and colony morphology when, following the initial

treatment with G-CSF, these cultures were refed with medium containing IL-6 plus sIL-6R (data not shown).

**Replace the paragraph spanning page 22, lines 6 to 18 with the following paragraph:**

The unusual appearance of GRgp(Y118F) cells in high concentrations of G-CSF is unlikely to be simply due to an increase in affinity of the Y118F receptor for G-CSF because this response is not observed in wild type cells treated with high levels of LIF, or in GRgp(278) transfectants treated with saturating levels of IL-6 (plus sIL-6R), G-CSF, or IL-6 (plus sIL-6R) plus G-CSF (Figure 2C). Furthermore the phenotype of GRgp(Y118F) cells in high levels of G-CSF was maintained when cells were simultaneously stimulated with G-CSF and IL-6 (plus sIL-6R). This observation excludes the explanation that the unusual ES cell morphology is due to a partial loss of self-renewal signals and suggests that the phenotype arises from hyperactivation of signals downstream of gp130. Collectively these data point to a key role for tyrosine 118 in downregulating gp130 signaling in ES cells.

**Replace the paragraph spanning page 22, line 21 to page 23, line 2 with the following paragraph:**

Since activation of SHP-2 may couple gp130 to the ERK pathway, we examined whether tyrosine 118 was also required for activation of ERK1 and ERK2 in ES cells. Activation of ERKs in GRgp130 transfectants treated with G-CSF or IL-6 (plus sIL-6R) was assessed by immunoblotting with an antibody specific for the phosphorylated (activated) forms of ERK1 and ERK2 (Figure 3). Basal levels of activated ERK were consistently detected in untreated cells following serum starvation. Increased ERK phosphorylation was observed in cells stimulated via the endogenous gp130 and GRgp(278) receptors. This was not evident on stimulation through the GRgp(Y118F) chimera. Reprobing with an antibody specific for the tyrosine phosphorylated form of STAT3, confirmed that both chimaeric receptors were effective at activating STAT3. These results establish that tyrosine 118 mediates activation of the ERK pathway in ES cells.

**Replace the paragraph spanning page 23, lines 6 to 19 with the following**

**paragraph:**

The capacity of GRgp(Y118F) to signal self-renewal implies that ERK activation is not required for the propagation of ES cells. To test this hypothesis, D027 cells were cultured in the presence of the specific MEK inhibitor, PD098059. A sub-saturating concentration of LIF (5 U/ml) was used in these experiments to increase the sensitivity of the assay to changes in self-renewal signaling. Surprisingly, treatment of ES cells with 3-25  $\mu$ M PD098059 did not inhibit self-renewal when compared with cells cultured in vehicle alone (~~Figure 4A~~). More surprisingly, in fact, the level of self-renewal increased in a dose dependent manner with the maximum level being achieved at 12-25  $\mu$ M. At concentrations greater than 50  $\mu$ M PD098059 the growth of ES cells was impaired, possibly as a result of some non-specific inhibitory effect of the drug, resulting in small undifferentiated colonies which stained positive for  $\beta$ -galactosidase by X-gal staining (~~data not shown~~).

**Replace the paragraph spanning page 23, lines 21 to 29 with the following**

**paragraph:**

To verify that ERK activation through gp130 was continuously suppressed by PD098059 in these long-term cultures, GRgp(278) cells were incubated for 48 hours with the inhibitor plus LIF and then stimulated through the chimaeric receptor with G-CSF. The immunoblot revealed that G-CSF dependent phosphorylation of ERK1 and ERK2 was progressively reduced from 3-12  $\mu$ M, and effectively blocked at 25  $\mu$ M PD098059 (~~Figure 4B~~). The continued proliferation of undifferentiated ES cells at inhibitory concentrations of PD098059 confirms that gp130-dependent activation of ERK1 and ERK2 is not required for the propagation of ES cells.

**Replace the paragraph spanning page 23, line 31 to page 24, line 7 with the following paragraph:**

The effect of PD098059 on self-renewal suggested that the inhibitor might alter the dose response of ES cells to LIF. Self-renewal of ES cells was assayed following treatment with 0.1-100 U/ml LIF either in the presence of 25  $\mu$ M PD098059 or vehicle (0.2 % DMSO) (Figure 4G). Treatment with PD098059 increased the level of  $\beta$ -galactosidase activity at all concentrations of LIF. This implies that the drug does not alter the dose dependency of ES cells but rather enhances their response to LIF. Significantly, PD098059 did not block the differentiation of ES cells in the absence of LIF.

**Replace the paragraph spanning page 24, lines 10 to 25 with the following paragraph:**

ES cell colony morphology and Oct-4 expression are reliable indicators of the undifferentiated phenotype, but do not establish that the cells are pluripotent. We therefore determined whether ES cells propagated in the absence of gp130-dependent ERK signalling have the capacity to incorporate into the developing embryo and differentiate appropriately. Cells were cultured at low density (1000 cells/cm<sup>2</sup>) for 48 hours in the presence of LIF plus 25  $\mu$ M PD098059, or in the absence of LIF. They were then re-fed with medium containing LIF but lacking the inhibitor for a further 24 hours before microinjection into mouse blastocysts. ZIN40 cells were used in this experiment, since they carry a nuclear localised  $\beta$ -galactosidase marker that is widely expressed in differentiated cell types. Staining of mid-gestation embryos for  $\beta$ -galactosidase revealed that ES cells treated with PD098059 contributed to chimaeras (Figure 5). However, cells cultured in the absence of LIF for 48 hours were incapable of colonizing the embryo (~~data not shown~~). This result confirms that gp130-dependent ERK activity is not required for maintaining the pluripotency of ES cells.



**Replace the paragraph spanning page 24, line 28 to page 25, line 13 with the following paragraph:**

We have previously established that activation of STAT3 is essential for gp130-dependent self-renewal of ES cells. To determine whether mutating tyrosine 118 affects this key regulator, activation of STAT3 was compared in GRgp(278) and (Y118F) transfectants. The acute stimulation of cells for 25 minutes with 30 fg/ml to 300 ng/ml of G-CSF did not reveal a significant difference between the levels of tyrosine phosphorylation of STAT3 induced by the chimaeric receptors (~~data not shown~~). However, signaling through the receptors was distinguished when the duration of the STAT3 signal was examined (~~Figure 6~~). Cells were stimulated with either G-CSF or IL-6 (plus sIL-6R) for 25 minutes, re-fed with cytokine free medium and then samples were collected at 40 minute intervals. A similar time course for the decay of phosphorylated STAT3 was obtained following stimulation through either the endogenous gp130 receptor or GRgp(278), with the signal being undetectable at 120 minutes. In contrast, the activation of STAT3 was sustained in G-CSF treated GRgp(Y118F) cells and could still be detected at 160 minutes. This result indicates that tyrosine 118 mediates a signal that normally attenuates the activation of STAT3.

**Replace the paragraph spanning page 25, line 17 to page 26, line 6 with the following paragraph:**

In order to investigate whether the prolonged activation of STAT3 influenced gene regulation in ES cells, we examined the expression of SOCS genes. These genes are rapidly induced by cytokines and encode proteins that can function as negative regulators of cytokine receptor function. SOCS-1 is a STAT3 target in M1 cells but this may not be the case in ES cells as we have not observed any increase in SOCS-1 expression in response to LIF (~~data not shown~~). In contrast, expression of SOCS-3 was transiently induced in ES cells stimulated either through the LIFR/gp130 complex with LIF or through the GRgp(278) chimaera (~~Figure 7~~). The peak level of observed expression occurred at 90 minutes after addition of cytokine, and returned close to uninduced levels by 3 hours. There was no induction of SOCS-3 transcripts in ES cells stimulated through a chimaeric

receptor, GRgp(Y126-275F), in which the four STAT3 docking sites have been eliminated by site directed mutagenesis. This result implies that the SOCS-3 gene is a target for STAT3 in ES cells. Significantly, following activation of the GRgp(Y118F) receptor the peak level of SOCS-3 expression obtained at 90 minutes was enhanced and in contrast to stimulation through the LIF receptor or GRgp(278), SOCS-3 mRNA levels remained elevated until at least 6 hours post-stimulation. It seems likely therefore that the prolonged activation of STAT3 results in enhanced expression of its target genes. This may underlie the shift in dose response to G-CSF observed for GRgp(Y118F) transfectants.

**Replace the paragraph spanning page 26, line 15 to page 27, line 1 with the following paragraph:**

IOUD2 ES cells, which carry a targeted integration of  $\beta$ geo into the *Oct4* locus were used to enable visualisation of undifferentiated cells by histochemical staining for  $\beta$ -galactosidase. Aggregates were formed in hanging drops by seeding 100 cells/20 $\mu$ l drop in the presence of 0, 25, 50, 75 or 100 $\mu$ m PD089059. Aggregates were maintained for 6 days, then transferred to gelatin-coated dishes and allowed to attach overnight. Cultures were then fixed and stained for  $\beta$ -galactosidase activity. In the absence of the Mek inhibitor, the embryoid bodies were well differentiated and very few *Oct4*  $\beta$ -galactosidase expressing cells were present in the outgrowths (~~Fig. 8, upper panel~~). In the presence of PD089059 however, the representation of undifferentiated  $\beta$ -galactosidase positive cells increased in a dose dependent fashion. At PD089059 concentrations of 75-100 $\mu$ m, the great majority of cells were undifferentiated (~~Fig. 8, lower panel~~). The numbers of undifferentiated cells in these conditions vastly exceeded these present in control cultures in the absence of MEK inhibitor, therefore this result is not simply due to ablation of cells. This finding indicates that ERK activation is critical to the process of embryoid body differentiation and that differentiation of stem cells can be prevented by reducing or abolishing Mek activity.